Trans generational effects of male age on son's mating success, Acps and sperm traits in *D. melanogaster*

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Abstract: Studies on human and nonhuman organisms have shown that the quality of gametes decreases with increasing of male age. Paradoxically, in many taxa, female prefer to mate with older males; however the adaptive significance of such preference is not clear until today due to lack of studies involving accessory gland proteins (Acps) and sperm traits. We used both cross sectional and longitudinal approaches to study male age effects on son's mating success, accessory gland proteins and sperm traits in *D. melanogaster*. It was noticed that in *D. melanogaster,* females of all age classes discriminated between sons of different male age classes and preferred to mate with sons of young males more frequently than with the sons of middle aged and old males. In pairwise mating, sons of young males showed a significantly greater courtship act compared to sons of middle aged and old males. In turn, females showed least rejection responses to the sons of young males than towards the sons of middle aged and old males. Further, sons of young males with smaller accessory glands, with a few larger main cells in their accessory glands, had produced greater quantities of Acps and were able to transfer significantly greater quantities of Acps and sperms to the mated females. As a result, females mated with them had greater fecundity and fertility than those mated with sons of middle aged or old males. Further, the sons of young males lived longer but females mated with sons of young males shorter life. Thus, our study suggests that with increasing of male age, the quality of gametes increases, resulting in reduction of Acps, and sperm traits of sons. Thus females of *D. melanogaster* obtain indirect genetic benefits by mating with young males.

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1. Introduction

Female use a variety of male phenotypes to select the potential mate in a given population (Grafen, 1990; Iwasa et al.,1991). Male age is one such honest indicator of male quality thereby females use it to select potential mates (Kokko and Lindstrom, 1996; Kokko, 1998; Price and Hansen, 1998; Beck and Powell, 2000; Beck et al., 2002). What is really important for females in such studies is not the quality of her mate but the quality of gametes produced with increasing age of males (Prokop et al., 2007).

Two main evolutionary theories namely good gene and antagonistic pleiotropic theories have been put forth to explain the mechanism responsible for the changes that occur in the quality of gametes with increasing age of males (Medawar, 1952; Williams, 1957). Both the theories are based on role of natural selection on mutation load which increases with age in males because somatic genetic quality of an individual may not change with age but the genetic mutation load of an individual can increase with age if germ line mutation probability increases with age. Good gene model believed that the forces of natural selection are weaker with age as a result newly arising lethal mutations in young organisms will be strongly selected against and will not be passed on to the next generation. Further the expression of the same mutations in older organism after the reproduction will not have a deleterious effect on the survival of the following generation. Antagonistic pleiotropy theory predicts that cellular damage and organismal ageing are caused by pleiotropic genes. Such genes may increase the chances of successful reproduction early in life but have deleterious effects later in life. Thus, two potentially opposing forces act on ageing in males. Thus the above studies suggest that there is a need to distinguish the male quality and gamete mutation load in models of sexual selection. Therefore studies involving sperm traits and offspring quality are essential in age based female mate preference.

Studies of human and non-human organisms have shown age related reductions in sperm quantity, quality, fertilization success and offspring fitness (Johnson and Gemmell, 2012). Therefore, given a choice of males, a female would prefer younger of the two competing males. This idea that younger males make better mates were first proposed by Hansen and Price (1995) and they provided four arguments 1) there are negative genetic correlations between early and late fitness components 2) males usually suffer a decrease in fertility with age 3) younger males

are better adopted to the current environment and 4) older males have accumulated more germline mutations. Further, the maintenance of female preference for younger and middle-aged males would be further strengthened if the reproductive fitness of females mating with older individuals declined owing to age related reduction in sperm quality through mutagenesis. Thus these studies suggest that the quality of offsprings decreases with increasing of males.

Paradoxically, females of many species prefer to mate with older males than young males (Sloter et al., 2006; Sartorius and Nieschlag, 2010). Even in human populations, studies with last few decades have shown that number of men between 35-49 years fathering children have increased markedly (Martin et al., 2003). However, it is not known whether old men fathering was due to female preference or simply to cultural changes in our own lifetime. Initially a series of verbal arguments followed by many experiments in diverse groups of taxa have been proposed for female preference for older males 1) if all else being equal among different male age classes females prefer older males because they have already demonstrated their capacity for survival. Therefore females mating with older males tend to have long living offsprings (Trivers, 1972). 2) Testing the such hypothesis requires to test the quality of gametes with age that is possible by testing the quality of offsprings produced by females mated with young, middle aged and old males. Further the effect of male age on progeny quality has rarely been tested (Radwan, 2003). Even such studies rarely followed individual males throughout their lives instead they assign discrete age classes that may potentially conceal some age related mating patterns, particularly if the age classes assigned do not span the life of the organism (Kidd et al. 2001; Hale et al., 2008; Hoikkala et al., 2008; Aitken et al., 2010; Dean et al., 2010). Although few research have undertaken longitudinal studies of male ageing but these are also inconclusive.

Model organisms such as *D. melanogaster* forms a good species to test the above hypothesis. It has provided valuable insights into the genetic contributions for many aspects of behavior, including those that occur during normal ageing (Boulianne, 2001; Grotewiel, 2005; Horiuchi and Saitoe, 2005).

Very few attempts have been made in species of *Drosophila* to understand the female preference for male age classes and offspring fitness (Avent et al., 2008; Prathibha et al., 2011; Somashekar and Krishna, 2011). However results of these studies were mixed and they did not test accessory gland and sperms traits in sons of different male age classes. In contrast to these studies females of *D. melanogaster* prefers to mate with young males more frequently than middle aged ones and old males (unpublished). Therefore, the present study has been undertaken in *melanogaster* to test the good gene model with the following aims:

1) Whether or not females of *D. melanogaster* discriminate sons of different male age classes. If so, do the quality of sons vary with increase in male age?

2) Whether or not the male age has any effect on son's sperm and Acps (accessory gland proteins) traits. If so, what is its relation to the number and size of main cells in the accessory gland and size of accessory glands.

3) Interrelations between duration of copulation, Acps and sperm traits, fecundity, fertility and longevity of females mated with sons of young, middle aged and old males.

2. Materials and Methods

The experimental stock was established from progenies of 50 isofemale lines of *D. melanogaster* that were collected in Mysore, India. These progenies were mixed together, and 20 males were placed together with 20 females in each culture bottle. Crosses were maintained at 22ºC±1ºC with 70% relative humidity on a medium of wheat cream agar. Flies were kept in a 12-h light:12-h dark cycle for three generations to acclimatize to laboratory conditions. Fourth-generation eggs were collected using Delcour's procedure (1969). Eggs (100) were then transferred into single vials. When adults began to emerge from these vials, virgin females and unmated males were isolated within 3 h of eclosion and maintained under the same laboratory conditions. The flies obtained from the *Drosophila* stock center at Mysore, India were also used. These flies were cultured and maintained as described above. Virgin females and unmated males were also obtained and maintained as noted above.

Assignment of age classes

For assigning age classes to males, the longevity (number of days from eclosion until death) of unmated males was determined by maintaining individuals in a vial until their death. Flies were transferred to a clean vial each week. Fifty replicates were performed, and the mean longevity was 65±2 days for the outbred population and 62±3 days for the Canton-S population. We also studied male mating activities from eclosion until death. Similar results were obtained for the two experimental stocks. Males showed low levels of activity on day 1 after eclosion. During days 2–54, however, males performed all mating activities and mated with females. After day 55, male mating activities declined greatly. "Based on these data, male age classes were defined as: young (2–3 days), middle-aged (4–28 days), and old (>28 days)."

The first set of emerged flies was aged 52–53 days (to obtain old males). When these flies were 25

days old the next set of flies was isolated and aged 27–28 days (to obtain middle-aged males). When these males were 25 days old (and the old set was 50 days old) the next set of flies was isolated and aged 2–3 days (to obtain young males). This procedure allowed us to culture and maintain young, middle-aged, and old males under the same conditions and to conduct experiments using these three sets of flies at the same time. Most (99%) cross sectional studies on ageing have used this protocol.

The unmated young, middle aged and old males were separately mated individually with 5-6 day old virgin females (obtained from a main culture bottle) to obtain offsprings (sons and daughters) and these offsprings were cultured in the same environment as described above until they were used in the present experiment.

Longitudinal study

The longitudinal study involves the offsprings obtained by females mated separately with same males at young, middle aged and old age. To study this, 2-3day old unmated males along with 5-6 day old virgin females were introduced into an Elens-Wattiaux mating chamber (1964) and observed for 1 hour. If mating occurred, we would allowed them to complete copulation. Then mated females were individually transferred once in 24hrs into a new vial containing wheat cream agar medium to obtain offsprings. Mated males were individually aspirated into a new culture vial containing wheat cream agar medium to allow them for ageing (27-28 days). When the mated males attained 27-28th days, they were individually allowed them to mate with 5-6 day old virgin females (obtained from main culture) and observed for 1 hour. If the pair didn't mate within an hour, they were discarded. If mating occurred, they were allowed to complete the copulation. Then twice mated males (1st mated at 2-3 days and 2nd mated at 27-28th days) were individually aspirated into new vials containing wheat cream agar media for ageing to 52-53 days. Mated females were transferred individually into a new vial containing food once in 24 hours to obtain offsprings (sons). When twice mated males attained 52-53 days, they were individually aspirated into Elens-Whattiaux mating chambers (1964) along with 5-6 days virgin females (obtained from the main culture bottle) and observed for 1 hour. If the pair remained unmated within 1 hour they were discarded. If mating occurred, we allowed them to complete the copulation and mated females were individually aspirated into a food vial to obtain offsprings. Sons obtained from females separately mated with the same male at young, middle aged and old age were used in the present experiment to assess the male age effect on son's fitness.

Female age classes were selected based on female rejection behavior toward courting males. Female rejection behavior was analyzed from day 2 to day 32 (females rarely mate after day 33) resulting in the following female age classes: young females (2–3 days), middle-aged females (17–18 days), and old females (32–33 days).

Effect of age on the son's mating success

To study female mating preferences for males of different ages, a virgin female obtained from the main culture bottle was placed in an Elens-Wattiaux mating chamber(1964) along with two unmated males. The virgin female was either sons of young, middle-aged, or old, whereas the two sons were of different male ages (sons of young with middle-aged males, sons of middle-aged with old males, or sons of young with old males). Each pair was observed for 1 h, and 50 trials were run for each age-class combination. Copulating pairs were aspirated from the mating chamber. The rejected sons in the female-choice experiment was also transferred to a new vial. We also measured wing length of 50 selected and rejected sons from each male age class combination, following the procedure of Hegde and Krihsna (1997). Separate experiments were run for both cross sectional and longitudinal studies. At the start of these experiments the effect of paint was tested by painting the thoracic region of one of the sons of same male age classes and allowing with a virgin female. The presence of paint did not affected mating probability (all groups, $p > 0.05$).

Effect of age on the son's accessory gland

Unmated sons of young, middle-aged, or old males were etherized, and accessory glands were dissected Medium A (Ashburner 1970).Tissues were fixed in 1 N HCl for 5 min. Accessory glands were photographed using a digital camera (Supporting figure S1). The shape of the accessory gland was generally that of an 's', 'c', or 'j'. Each gland was therefore divided into smaller areas consisting of triangles, trapeziums, and rectangles (Supporting figure S1). The area of each geometrical form was then calculated Ravi Ram and Ramesh (2002), and the sum of these areas represented the size of the gland (cm^2). The actual area of the gland was calculated by dividing these values by the magnification. Soon after taking these photographs, accessory glands were transferred into 2% lactoaceto orcein stain for 20 min. Glands were then gently opened with fine entomological needles and squashed between a glass slide and a coverslip. Acetic acid (45%) was used to spread the main cells of the accessory glands into a single layer. The total number of main cells in each accessory gland was counted, and main-cell sizes were measured. For cell-size measurements, the length of 50 randomly selected main cells was measured using a micrometer. Fifty accessory glands were analyzed for sons of each male age classes (young, middle-aged,

and old). Separate experiments were carried out for cross sectional and longitudinal studies.

Effect of age on the son's quantity of Acps

Accessory glands were dissected from sons of young, middle-aged, or old males in insect saline using entomological needles. Males of each age class were either unmated or had recently copulated (<5 min before they were sacrificed). Glands were fixed in 95% ethanol. Fixed glands were placed on a glass slide, and the membrane was removed using a fine needle and a stereomicroscope (Supporting figure S2). The isolated secretions were washed in methanol/chloroform (1:1) and dried at 37°C for 15 min. Approximately 100 µL sample buffer (0.625 M Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, and 10% glycerol) was added to each sample to dissolve the glands and secretions. Twenty pairs of accessory glands from each age class (10 mated and 10 unmated males for each trials) were collected and, total Acp was estimated using the Bradford method (1976). Fifty trials were run for sons of each male age classes (young, middle aged and old). Separate experiments were carried out for cross sectional and longitudinal studies.

Bradford method

Approximately 50 µL of Acps (obtained as described above) were mixed with 5 mL Bradford reagent, which was generated by adding 100 mg Coomassie Brilliant Blue G-250 (in 50 mL 95% ethanol) to 100 mL 85% phosphoric acid and then diluting the mixture to 1 L with distilled water. The solution was allowed to stand for 5 min to develop color. The quantity of proteins present in each sample was determined by measuring optical density at 595 nm of the solution using a spectrophotometer. Bovine serum albumin was used as the standard. Fifty trials were run for sons of each male age classes (young, middle aged and old). Separate experiments were carried out for cross sectional and longitudinal studies.

Effect of male age on son's courtship activities

A virgin female (5–6 days old) and a male (young, middle-aged, or old) were placed in an Elens-Wattiaux mating chamber (1964), and observed for 1 h. Pairs that did not mated were discarded. If mating occurred, the duration of copulation was recorded. The courtship acts of son's tapping, scissoring, vibrating, licking, and circling were quantified, as were the female rejection behaviors of ignoring, extruding, and decamping. These behaviors were quantified according to Hegde and Krishna (1997). Tapping is when a son's initiates courtship with a foreleg motion. He partially extends and simultaneously elevates one or both forelegs and then strikes downward, thus bringing the ventral surface of the tarsus in contact with the partner. Scissoring occurs during the interval between wing vibrations when a courting male opens and closes both wings using a scissor-like movement. Licking is when a courting son's positions himself closely behind the female, extends his proboscis, and licks her genitalia. Circling is when a sons, after posturing at the side or rear of a non-receptive female, faces her as he moves. Sometimes he moves to face her and then retraces his path to the rear. Other times he moves around her in a full circle. The female rejection behavior of ignoring is when a courted, non-receptive female continues activity and apparently ignores the actions of the sons. Extruding is when a non-receptive female presses the vaginal plates together, contracts certain abdominal muscles, and apparently relaxes other muscles. Decamping is when a non-receptive female attempts to escape by running, jumping, or flying away from the courting sons.

The behavior of males and females engaged in courtship were recorded simultaneously by two observers for 1 h. One observer quantified female behavior, and the other quantified male behavior. Fifty pairs that successfully mated pair from each age class were used. Separate experiments were run for both cross sectional and longitudinal studies.

Eggs and progeny produced by female that mated with sons of different male age classes

Individual mated females were collected and placed into a vial. Every 24 h a female was placed into a new vial, a procedure that was repeated until the death of the fly. The total number of eggs and emerged progeny were counted. Separate experiments were performed for both cross sectional and longitudinal studies.

Effect of male age on son's copulation duration, quantity of Acps, and sperm count

A unmated sons of young, middle-aged, or old unmated male was placed in an Elens-Wattiaux mating chamber (1964), with a virgin female (5–6 days old). The pair was observed for 1 h. Pairs that did not mate were discarded. If mating occurred, the copulation duration was recorded. Soon after mating, the reproductive organ of the female was dissected in 20 µL Beadle-Ephrussi Saline (128.3 mM NaCl, 4.7 mM KCl, 23 mM $CaCl_2$) (Ephrussi-Beadle 1936). Because sperm could dissociate into the solution, 20 µL lactoaceto orcein was added to the slide without draining the saline. The number of sperm was then counted using an Olympus CX21 microscope. The quantity of Acps was measured for the mated sons as described above. Fifty trials were run for sons of each male age classes (young, middle aged and old). Separate experiments were carried out for cross sectional and longitudinal studies.

Statistical analysis

We applied Generalized Linear Model with a binomial link function to age based female mate

preference The mating success of the sons of younger male was the dependent variable his age class and the age class of the alternative mate were the fixed factors and the differences in wing length between two competing males was the covariant. One way ANOVA followed by Tukey's post hoc test carried out all the above parameters studied. In addition to this, Principle component of analysis to son's and female courtship activities as also been carried out survival curve was calculated for longevity of females mated to sons of different male age classes. Two functions that are dependent on time are of particular interest: the survival function and the hazard function. The survival function S(t) is defined as the probability of surviving at least until time t. The hazard function h(t) is the conditional probability of dying at time t having survived until that time. The graph of S(t) against t is called the survival curve. The Kaplan-Meir method was used to estimate this curve from observed survival times without assuming an underlying probability distribution. Two survival curves were compared using a statistical hypothesis test called the log-rank test, which is used to test the null hypothesis that there is no difference between survival curves, i.e., the probability of an event occurring at any point of time is the same for sons of each male age classes.

3.Results

For both cross sectional and longitudinal studies of *D. melanogaster*, virgin females of all ages(young, middle aged and old) preferentially mated the sons of younger male in female choice experiment (Table 1). Significant variation was noticed in mating success between sons of different male age classes. However variation in mean wing length between selected and rejected sons in these experiments were not significant (Table 1).

Sons of young males showed the highest frequency of tapping, scissoring, circling, and licking behaviors toward females, whereas sons of old males showed the lowest levels of courtship behaviors (Table 2a). In turn, female generally showed fewer rejection responses (decamping, ignoring, and extruding) to sons of young males than to sons of middle-aged and old males. Similar results were found in both the studies. Significant variation was found in sons of courtship activities between males of different ages. In addition, there was significant variation in female decamping behavior toward sons of different male ages, but similar levels of ignoring and extruding behaviors were presented to sons of all male age classes. Sons of young males exhibited significantly more courtship activities than sons of middle-aged males, and sons of middle-aged males courted more than sons of old males Principle component analysis revealed that in son's tapping and scissoring had greater influences on mating success than licking and circling. While in female decamping and extruding had greater influences on mating success than ignoring and extruding (Table 2b).

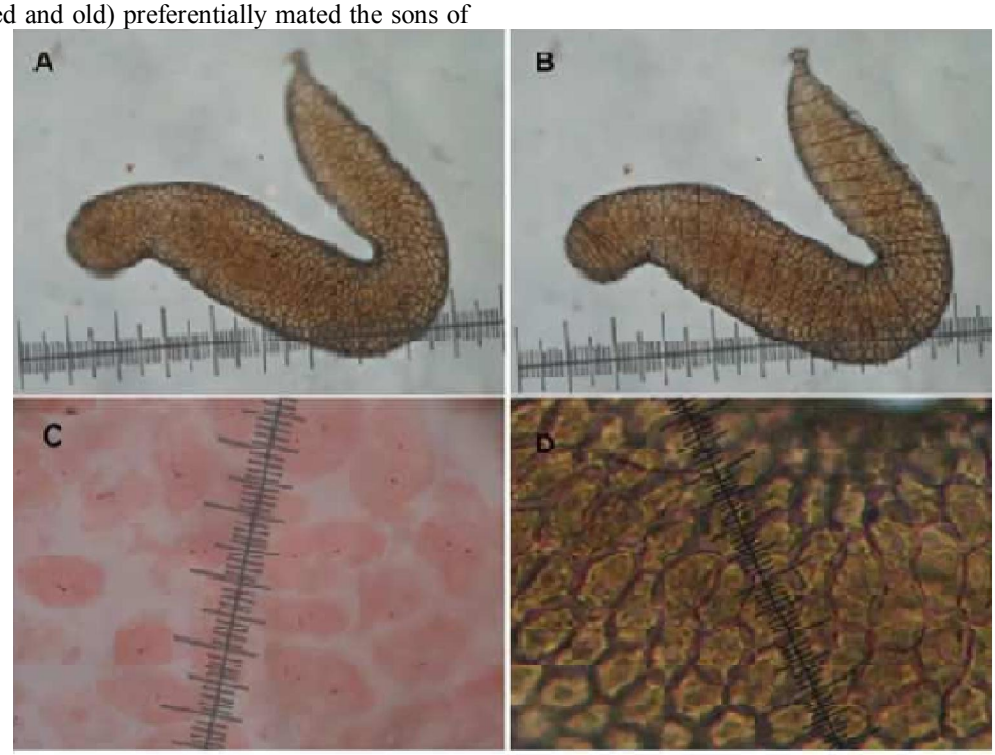

Figure S1. Measurement of cell number, cells size and gland size. (a. Accessory gland lobe. b. Marked accessory gland lobe for measuring the size of the gland. c and d. Measurement of cell size of accessory gland lobe.

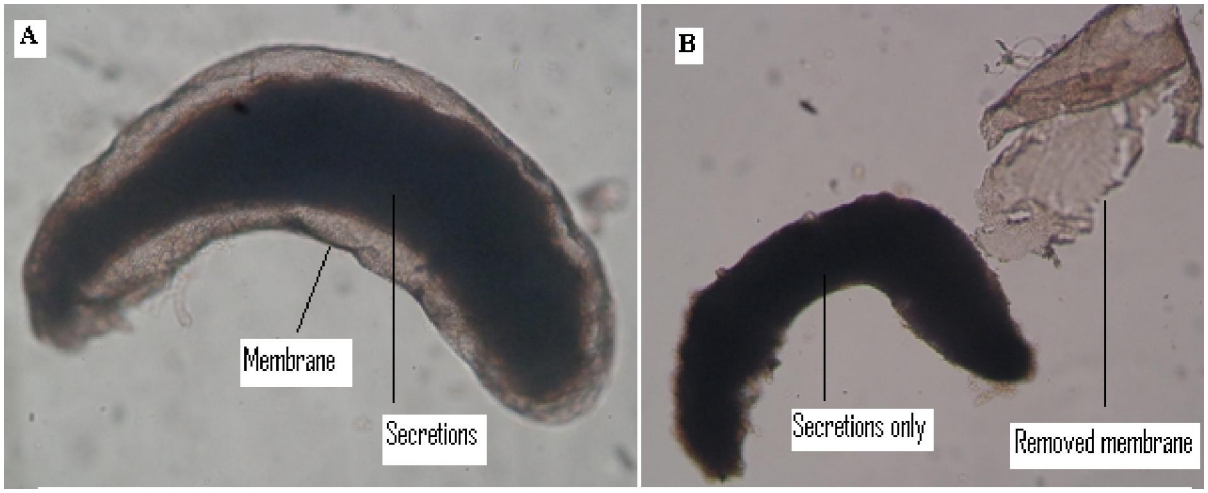

Figure S2. Measurement of quantity of Acps using the secretion alone (a. Secretion with membrane. b. Secretion without membrane).

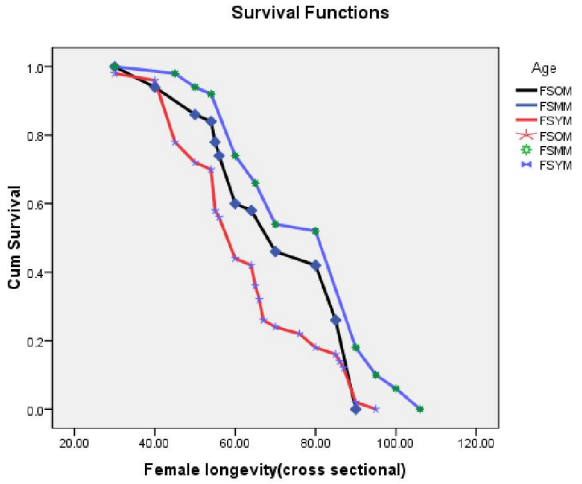

Figure 1a. Survival curve of females mated to sons of different male age classes in cross sectional studies of *D. melanogaster*.

FSOM, Female mated to sons of old male, FSMM, Female mated to sons of middle aged male, FSYM, Female mated with sons of young male.

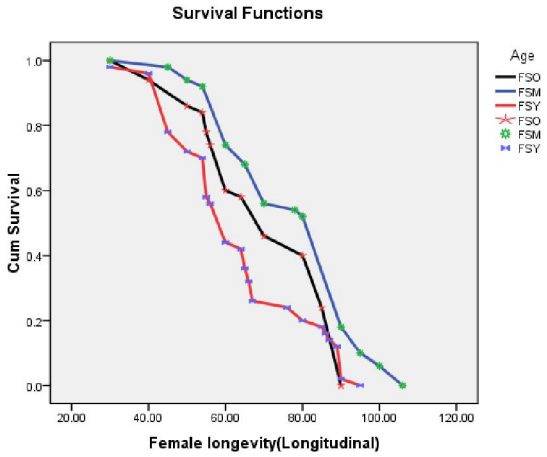

Figure 1b. Survival curve of females mated to sons of different male age classes in longitudinal studies of *D. melanogaster*.

FSOM, Female mated to sons of old male, FSMM, Female mated to sons of middle aged male, FSYM, Female mated with sons of young male.

Table 1. Female mate preference for sons of different male age classes in *D. melanogaster* (N=50; df. 1).

	Female age	Sons		Wald chi-square (mating success)	Sig. level
		SYM	**SOM**		
CSS	Young	34(68%)	16(32%)	3.15	0.078
	Middle	38(76%)	12(24%)	6.08	0.014
	Old	40(80%)	10(20%)	7.71	0.005
LS	Young	35(70%)	15(30%)	4.51	0.0032
	Middle	39(78%)	11(22%)	7.08	0.008
	Old	41(81%)	09(18%)	7.91	0.005
		SMM	**SYM**		
CSS	Young	19(38%)	31(62%)	1.56	0.21
	Middle	18(36%)	32(64%)	1.91	0.167
	Old	14(28%)	36(72%)	0.274	0.601
LS	Young	18(36%)	32(64%)	1.92	0.163
	Middle	16(32%)	34(68%)	2.97	0.086

	Old	15(30%) 35(70%)	4.22	0.041
		SOM SMM		
CSS	Young	16(32%) 34(68%)	2.92	0.083
	Middle	15(30%) 35(70%)	4.12	0.040
	Old	14(28%) 36(72%)	1.270	0.600
LS	Young	12(24%) 38(76%)	6.08	0.014
	Middle	14(28%) 36(72%)	3.71	0.052
	Old	12(24%) 38(76%)	6.07	0.014
	Female age	**Sons**	**Wald chi-square (mating success)**	**Sig. level**

SYM, sons of young male, SMM, sons of middle aged male, SOM, sons of old male.
CSS, Cross sectional study; LS, Longitudinal study.

Table 2a. One way ANOVA of male age effect on son's courtship activities of *D. melanogaster*

	Son's courtship activities	**Mean ± SE**			
		SYM	SMM	SOM	**F value**
CSS	Tapping	13.24±0.17	11.32±0.34	4.96±0.39	11.89**
	Scissoring	4.11±0.49	2.21±0.54	3.51±0.61	9.59**
	Circling	4.64±0.21	3.72±0.17	2.64±0.12	17.30*
	Licking	3.82±0.22	3.58±0.20	2.54±0.40	4.40*
LS	Tapping	12.24±0.57	8.84± 0.14	4.56±0.29	14.79**
	Scissoring	5.31± 0.47	4.01±0.44	2.11±0.39	8.397**
	Circling	1.74± 0.02	0.84±0.03	0.42±0.02	15.33**
	Licking	4.82± 0.21	3.58±0.21	3.50±0.41	4.48*
	Female rejection responses				
CSS	Decamping	2.62±0.17	3.12±0.34	3.94±0.39	24.80**
	Ignoring	1.88±0.54	2.58±0.49	3.61±0.67	1.197^{NS}
	Extruding	1.48±0.18	1.84±0.07	2.68±0.06	1.121^{NS}
LS	Decamping	3.32±0.28	5.32±0.21	6.44±0.17	16.28**
	Ignoring	2.08±0.19	3.48±0.11	3.91±0.25	1.197^{NS}
	Extruding	1.08±0.11	1.94±0.17	2.38±0.16	1.804^{NS}

SYM, sons of young male, SMM, sons of middle aged male, SOM, sons of old male.
**** Significant at $p < 0.001$, * Significant at $p < 0.005$. NS- Insignificant at $p > 0.05$.**
CSS, Cross sectional study; LS, Longitudinal stud

Table 2b. Principal component analysis of male age effect on son's courtship activities of *D. melanogaster*.

	Total Variance Explained			
	Son's courtship activities	**Initial Eigenvalues**		
		Total	% Variance	Cumulative %
CSS	Tapping	1.621	40.522	40.522
	Scissoring	1.052	26.296	66.818
	Circling	0.791	19.772	86.590
	Licking	0.536	13.410	100.000
LS	Tapping	1.428	35.700	35.700
	Scissoring	1.160	29.009	64.709
	Circling	0.770	19.255	83.964
	Licking	0.641	16.036	100.000
	Female rejection responses			
CSS	Decamping	1.606	53.525	53.525
	Ignoring	0.874	29.131	82.656
	Extruding	0.520	17.344	100.000
LS	Decamping	1.394	46.456	46.456
	Ignoring	1.017	33.895	80.351
	Extruding	0.589	19.649	100.000

CSS, Cross sectional study; LS, Longitudinal study

Table 3. Male age effect on son's accessory gland structure and quantity of Acps in cross sectional and longitudinal studies of *D. melanogaster*

		Accessory gland parameters		
	Main cell number of accessory gland (N =50; df=2,147)	**Main cell size of accessory gland (in mm) (N =50; df=2,147)**	**Accessory gland size (in cm^2) (N =50; df=2,147)**	**Quantity of Acps in µg/pair of gland (unmated) (N =50; df=2,147)**
CSS SYM	2066±45[a]	0.0058±0.00020[a]	0.369±0.019[a]	20.12±0.14 [a]
SMM	2312±83[b]	0.0046±0.00015[b]	0.381±0.028[b]	19.23±0.13[a]
SOM	2510±68[c]	0.0044±0.00019[c]	0.408±0.024[c]	18.52±0.12[b]
F value	11.57**	19.11**	29.502**	42.20**
LS SYM	2120±45[a]	0.0061±0.00020[b]	0.428±0.024[b]	20.38±0.17[b]
SMM	2612±83[b]	0.0055±0.00015[a]	0.439±0.019[a]	19.68±0.19[b]
SOM	2920±68[c]	0.0052±0.00019[a]	0.451±0.028[a]	18.76±0.15[a]
F value	11.98**	19.86**	31.76**	42.98**

SYM, sons of young male, SMM, sons of middle aged male, SOM, sons of old male.
Different letter in the superscript indicates significance at 0.05 levels by Tukey's post hoc test
** Significant at $p < 0.001$.
CSS, Cross sectional study; LS, Longitudinal study

Table 4. Mae age effect on son's copulation duration and son's transferred quantity of Acps and sperm to the mated female in cross sectional and longitudinal studies of *D. melanogaster*

		Accessory gland parameters			
	Copulation duration (in minute) (N =50; df=2,147)	Quantity of Acps (mated) (in µg/pair of gland)	Transferred[β] quantity of Acps (in µg/pair of gland)	Fecundity (in numbers) (N =50; df=2,147)	Fertility (in numbers) (N =50; df=2,147)
CSS SYM	21.28±0.98	16.30±0.19	3.82±0.12[a]	220.23±6.11[b]	110.11±2.10[ab]
SMM	22.88±0.98	16.45±0.18	2.78±0.11[b]	170.23±6.14[a]	95.12 ±1.14[b]
SOM	23.00±0.98	16.61±0.17	1.91±0.10[c]	157.16±6.12[b]	73.23±3.24[a]
F value	1.21[NS]	2.15[NS]	24.63**	19.11**	29.502**
LS SYM	19.31±0.76	15.94±0.12	3.52±0.11[a]	399.23±6.11[a]	229.11±2.10[b]
SMM	20.79±0.67	15.81±0.14	2.12±0.11[b]	264.23±6.14[b]	129.12 ±1.14[a]
SOM	21.32±0.73	15.61±0.16	1.01±0.10[c]	219.16±6.12[c]	119.23±3.24[a]
F value	0.18[NS]	1.91[NS]	16.03**	56.86**	42.76**

SYM, sons of young male, SMM, sons of middle aged male, SOM, sons of old male.
**** Significant at $p < 0.001$, * Significant at $p < 0.005$. NS- Insignificant at $p > 0.05$.**
CSS, Cross sectional study; LS, Longitudinal study
(Difference in quantity of Acps between mated and unmated son was considered as a transferred quantity of Acps: this data was used to calculate mean and SE of **transferring quantity of Acps**.
Ex. Quantity of Acps of Unmated trials, 1, 2,3,....,50
Quantity of Acps of Mated trials, 1,2,3,…..,50
Difference in quantity of Acps between mated son and unmated son, 1,2,3,….,50)

Table 5. Overall tests of the equality of survival times of different male age classes in cross sectional and longitudinal studies of *D. melanogaster*.

CS	Log Rank (Mantel-Cox)	4.334	1	0.037
	Breslow (Generalized Wilcoxon)	5.980	1	0.014
	Tarone-Ware	5.312	1	0.021
LS	Log Rank (Mantel-Cox)	27.206	2	0.000
	Breslow (Generalized Wilcoxon)	23.000	2	0.000
	Tarone-Ware	25.070	2	0.000

Concerning accessory gland traits of unmated sons of different male age classes, the number of main cells and the size of the son's accessory gland increased with age of male, whereas the quantity of Acps and the size of the main cells of son's decreased with age of male(Table 3). Significant variation was noticed in above traits between sons of male age classes. Results were similar for cross sectional and longitudinal studies.

The son's copulation duration generally increased with increasing age of male. In contrast, the son's quantity of Acps, the amount of son's Acps and

sperm transferred to the mated female and the fecundity and fertility of the mated female decreased with the age of the male(Table 4). The amount of son's Acps transferred to the mated female as well as the fecundity and fertility of the mated female varied significantly sons of different male age classes. No significant variation in copulation duration was measured between sons of different male age classes. The quantity of transferred sons of Acps and sperms correlated positively with sons of copulation duration and the fecundity and fertility of the mated female. Results were similar for both studies.

Figure 1a and b and Table 5 revealed that females that mated with a son's middle-aged or old male lived significantly longer than females that mated with a son's young male.

4. Discussion

Our study provides a strong evidence that females of *D. melanogaster* discriminate between sons of different male age classes (Table 1). Given a choice of selecting between the sons of two male age classes, virgin females of all the three age classes (young, middle aged and old females) preferred sons of youngest of the two competing males. This result was found to be similar in both cross sectional and longitudinal studies. Our results supports the female discriminating sons of different male age classes in *D. bipectinata* and *D. ananassae* (Somashekar and Krishna, 2011; Prathibha et al., 2011) and they also found that female had preference for old males. However females of *D. pseudoobscura* did not discriminate between sons of young and old male (Avent et al., 2008). This shows the existence of species specific difference in female preference for sons of different male age classes. Insignificant difference in the wing length of selected and rejected sons in female mate choice experiment suggests that observed discrimination in female mate preference for sons of different male age classes was not influenced by son's body size. Why do females of *D. melanogaster* prefer to mate with sons of young males? Possible reasons include: 1) sons of young males may be more vigorous or active during courtship thereby convincing the female that sons of young males will be quicker to copulate : 2) female show less rejection behavior for preferred sons of different male age classes: 3) females obtain greater quantity of Acps and sperms by mating with sons of young males : 4) in resource free mating, females expert direct fitness benefits, i.e. a greater number of eggs and progeny from mating with sons of young males. Table 2a revealed that in *D. melanogaster* sons of young male performed greater number of courtship acts with in a short period compared to sons of middle aged and old males. Through these courtship acts the sons of young males had conveyed the chemical and mechanical information faster to courting female than to those sons of middle aged and old males. In turn, females showed least rejection responses to the sons of young males than to the sons of middle aged or old males (Table 2a and b). This suggests that the sons of young males were more vigorous during courtship thereby he could convince the female faster to mate.

In contrast to this in *D. bipectinata* and *D. ananassae* (Somashekar and Krishna, 2011; Prathibha et al., 2011) the sons of old male performed greater courtship acts to courting female and could convince the male for mating faster than that of the sons of middle aged and young males. These suggest that male age effect on offspring traits differ in different species of *Drosophila*.

A careful observation of Table 3 reveals that sons of young males with shorter accessory glands having fewer larger main cells in their accessory glands are able to produce significantly greater quantities of Acps while sons of the old males with larger accessory glands having numerous shorter main cells in their accessory glands are able to produce a lesser quantity of Acps. This shows that the occurrence of male age effects on the son's accessory glands structure and quantity of Acps in *D. melanogaster*. Ravi Ram and Ramesh (2001) who while studying in *D. nasuta* have found a lack of relationship between the main cells number and the quantity of Acps production. However, they did not measure the size of main cells of accessory glands.

Table 4 shows that in *D. melanogaster* although sons of young males copulated shorter time they had transferred significantly greater quantity of Acps and sperms to the mated females than that of the sons of middle aged and old males. Further females who mated with the sons of young males had produced the greater number of eggs and progenies than those that of females mated with either sons of middle aged or old male. Price and Hansen (1998) who while studying in *D. melanogaster* has found a reduction in offspring viability and mating ability in sons of 5-week old males but the variation was insignificant, suggesting that the quality of offsprings decreased with increasing of male age. In contrast to this in *D. bipectinata* and *D. ananassae* female who mated with sons of old male had laid greater number of eggs and produced greater number of progenies than that of the female mated with either sons of middle aged and young males (Somashekar and Krishna, 2011; Prathibha et al., 2011). However they did not studied Acps and sperm traits in sons of different male age classes.

Our results in *D. melanogaster* support earlier a negative effect of male age on offspring fitness in

nonhuman studies (Garcıa et al. 2009; Garcia-Palomares et al., 2009). Even in studies on humans, increasing male age has been linked to an increased risk of offspring disorders such as autism, Down's syndrome, epilepsy and schizophrenia (Sartorius and Nieschlag, 2010). Results obtained in our study in *D. melanogaster* did not support the good gene hypothesis proposed by Kokko and Lindstrom (1996), and our results which suggest that offspring of old males do not always bear good quality genes. Further, little empirical evidence is also available in which male age has a positive effect on egg viability (Pervez, 2004). The declines in fertilization success observed in sons of middle aged and old males in *D. melanogaster* may have arisen through differences in the transferred quantity of sperms to mated females. Very few studies have specifically tried to understand how any potential decline in sperm traits with age might affect offspring viability. These studies have revealed that the DNA in the male germline increases the mutational load carried by the embryo (Hansen and Price, 1999; Crow, 2000; Aitken et al., 2004); even in humans, it has been shown that embryo quality decreased with increasing age of males (Simon et al. 2006). Further, it is also shown that older males have more DNA lesions in the germline than middle-aged males (Velando et al., 2011).

Most of these studies exploring age related variations in offspring qualities have used only cross sectional approaches. Therefore such studies do reveal male age influences on sperm traits and offspring fitness. In the present study, we found similar results of the decline in offspring quality with increasing of male age in both cross sectional and longitudinal studies, which revealed that male age related changes in sperm traits and offspring fitness are found to be similar in both studies. Therefore the results of earlier works using cross sectional approaches for male age effects on offspring fitness are also valid.

Studies on *Drosophila* and other insects have shown that mating helps the male to increase the fitness by reducing the fitness of the female i.e. mating reduces the female longevity (Wolfner, 2002). Our study on *D. melanogaster* has also shown that females who mated with sons of young males lived shorter than females who mated with sons of middle aged and old males (Figure 1a, b and Table 5). This may be attributed to longer duration of copulation in females who mated with the sons of young males, which resulted in transferring greater quantity of accessory gland proteins to the mated females. Our study also confirms earlier studies of the relations between duration of copulation and transferred quantity of ejaculates substances (Thornhill and Alcock, 1983).

Conclusion

Our studies in *D. melanogaster* demonstrate that the quality of offsprings decreases with increasing of male age. Females of *D. melanogaster* discriminate sons of different male age classes and they preferred to mate with sons of young males. This preference could be due to greater courtship acts during mating and also to greater investment in mating by the sons of younger males. As a result, females who mated with sons of young males obtain significantly greater quantity of sperms and Acps; thereby the females mating with sons of young males gain increased eggs and progeny production but reduced female longevity. Thus, these studies suggest that in *D. melanogaster* quality of gametes varies with male age and it decreases with increasing of male age, resulting in reduction of Acps and sperm traits of sons. Thus females of *D. melanogaster* obtain the indirect genetic benefits by mating with young males.

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